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The Mechanism of 8-Cl-cAMP Action

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8-Chloroadenosine 3', 5'-monophosphate (8-Cl-cAMP) is a potential new anticancer agent, but its mechanism of action is not clearly defined. In this work we have studied the effect of various heat inactivated and heat untreated human sera in the absence or in the presence of a nonspecific phosphodiesterase (PDE) inhibitor, IBMX, or of nucleoside transport inhibitor and cGMP-specific PDE inhibitor dipiridamole (DP), or of inosine-monophosphatedehydrogenase (IMPDH) inhibitor, tiazofurin, (T), on the antiproliferative 8-Cl-cAMP action towards two human malignant cell lines, K562 and HeLa cells, *in vitro*. Cell survival was determined 72 hrs after the agents action, using MTT assay. The results obtained, indicated the similar inhibitory effect of 8-Cl-cAMP on HeLa cell survival in the presence of four different heat untreated human sera (IC_{50}= 4-4.8 μM). Serum heat inactivation caused decrease in 8-Cl-cAMP antiproliferative action depending on the blood donor (IC_{50}= 23 μM, 15 μM, 19 μM, and 9 μM) and suggesting that some thermolabile ingredient(s) present in sera is involved, at least partially, in the induction or permittance of antiproliferative 8-Cl-cAMP action. K562 Cells were not as much resistant to 8-Cl-cAMP as HeLa cells, or mouse melanoma B16 cells; in the presence of heat untreated FBS, IC_{50}=16 μM, while for B16 cells IC_{50} was 8 μM. Different human sera show different effect on 8-Cl-cAMP action on K562 cells: IC_{50}= 7.5 μM and 16.5 μM. In the presence of heat inactivated human sera 8-Cl-cAMP IC50 concentrations were higher, with relevant mutual differences. The effect of different sera on 8-Cl-cAMP action was only partly abrogated in the presence of a nonspecific PDE inhibitor, IBMX, suggesting that the serum PDE action is one of the various factors contributing to the induction of 8-Cl-cAMP antiproliferative action. Nucleoside transport inhibitor and cGMP-specific PDE inhibitor dipiridamole inhibited the antiproliferative 8-Cl-cAMP action to HeLa and K562 cells. Tiazofurin and 8-Cl-cAMP acted as antagonists on HeLa, but not on K562 cells.

Key Words: 8-Cl-cAMP, Human serum, HeLa cells, IBMX, Dipyridamol, PDE

Receptors of cyclic AMP are regulatory subunits of cAMP-dependent protein kinase A, (PKA) (1). This kinase exists in its inactive form as a tetramer composed of an R subunit dimmer and two monomeric catalytic subunits, C. In the holoenzyme form R_2C_2 the C subunits are inactivated through their association with R subunits. The binding of two molecules of cAMP to each R subunit led to of C subunits dissociation which are then catalytically active for phosphorilation of target proteins (2).

Most tissues contained in general two PKA isosymes, type I (PKA I) and type II (PKA II), whose R subunits RI and RII were different while C subunits were identical (3). The higher expression of mRNA for RI subunit of PKA, in comparison with surrounding normal tissue, was found in many distinct human malignancies such as renal (4), gastric (5) and colorectal cancer tissue (6). Significantly higher levels of typeI/typeII PKA ratio (7) and an elevation in PKA activity was found in primary breast carcinoma as compared with normal breast tissues (8). No change in RI/RII ratio (9) and an increase in RI have been reported, too (10). Further, the higher expression of RI in comparison with RII of PKA was found in many malignant hormone-dependent MCF-7, T47 D and ZR-75-D, or hormone-independent MCF-7*ras*, MDA-MB-231 and BT-20 breast cancer cell lines, in WiDr; LS-174T and HT-29 colon carcinoma, in A549 lung carcinoma cell lines, in FOG and U251 gliomas and HL-60; K562; K562*myc* and MOLT-4 leukemia cell lines (11-15). It was demonstrated that an antisense phosphorothioate oligodeoxynucleotide (S-oligodeoxynucleotide) against RIa mRNA of

PKA in dose-dependent fashion inhibited proliferation of breast carcinoma cell, MCF-7 cells (16). The same was found on human colon carcinoma cells, LS-174T cells, as well as of human neuroblastoma cells, SK-N-SH cell line (15) and HL-60 promyelocytic leukemia cells (17) the decrease in tumour volume in many patients with breast cancer treated with tamoxifen was associated with a correspondant decrease in the levels of mRNA for the RI subunit of PKA (18). These findings (see reviews, Cho-Chung) (19, 20) indicated the possibility that an overexpression of RI, in comparison with RII, could be a general phenomenon associated with neoplastic cell transformation and maintenance.

Although the antiproliferative action of 8-Cl-cAMP was clearly documented on human colon and lung cancer cells (21, 22), leukemia cells (23), human glioma cells (24), Ki-ras-transformed rat fibroblasts (25) and on many other cell lines (11, 12), as well as in several experiments *in vivo* on human xenograft bearing nude mice (26, 27), the exact molecular mechanism of 8-Cl-cAMP antiproliferative action was not completely understood. In general, it is believed that 8-Cl-AMP exerts its antiproliferative action through modulation of intracellular levels of the two isoforms of PKAI and PKAII that led to the induction of transcriptional factors that restore normal gene regulation in cancer cells (28). 8-Cl cAMP appears to bind to the RII regulatory subunit of PKAII with high affinity to site B, but with low affinity to site A, preventing activation and release of the two catalytic subunits C. The 8-Cl-cAMP binds also to RI, site A and B, with moderately higher affinity facilitating C subunit dissociation and downregulation of PKA I (20). Also, it was postulated, that the effect of 8-Cl-cAMP is mediated through its catabolic product, 8-Cl-adenosine, and that 8-Cl-cAMP serves as pro-drug of its active metabolite 8-Cl-adenosine (29-33). Although earlier studies indicated that 8-Cl-adenosine acted as an inhibitor of DNA polymerase (22, 34), recent experiments (35), indicated that 8-Cl-adenosine can also modulate the expression of PKA isosymes, not through its binding to RI or RII but rather by reducing the C subunit expression. Insufficient presence of C subunit eventually led to the degradation of highly unstable free RI.

As phase I of the clinical trial of 8-Cl-cAMP had started (33, 36, 37), and there are some indications on the potential use of this drug for cancer therapy, the aim of this work was to see whether any differences in the 8-Cl-cAMP action in various fresh or heat-inactivated human sera could be found; it was also investigated whether the number of target cells influences the extent of 8-Cl-cAMP action and which of the three investigated cell lines is the most sensitive to the drug action in the presence of fresh FBS and what is the role of phosphodiesterases (PDE) inhibitor, IBMX, or nucleoside transport inhibitor and preferential cGMP-dependent PDE inhibitor, dipiridamole, DP, on 8-Cl-cAMP action on investigated cell lines.

Materials and Methods

Cell line, medium and chemicals. Experiments were done on the K562 human myelogenous leukaemia cell line, on human cervix carcinoma, HeLa cells and on mouse melanoma B16 cells. RPMI 1640 cell culture medium and fetal bovine serum (FBS) were products of Gibco (Paisley, Scotland, U.K.). Drugs 8-Cl-cAMP and tiazofurin were kindly obtained from ICN Pharmaceuticals, (USA). 3-isobutyl-1-methylxanthine, IBMX and dipyridamole were purchased from Behring (Germany). MTT, 3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyl tetrazolium bromide was provided by Sigma (St. Luis, MO, U.S.A). It was prepared as a 5 mg/ml stock in phosphate buffer saline pH 7.2, and filtered through milipore filter, 0.22 μm, before use.

Cell culture: Human cervix carcinoma HeLa cells, as well as mouse melanoma B16 cells were maintained as a monolayer culture, while human myelogenous leukemia, K562 cells were grown as a suspension culture, in the same nutritient medium (RPMI 1640 supplemented with l-glutamine (3 mmol/L), streptomycin, and garamycin (100 μg/mL, each), and 25 mM Hepes, adjusted to pH 7.2 by bicarbonate solution, with 10% heat inactivated foetal bovine serum, FBS). The cells were grown at 37°C in 5% CO_2 and humidified air atmosphere by twice weekly subculture.

Treatment of HeLa and B16 cells. HeLa cells, human cervix carcinoma cells, were seeded in triplicate into 96-well microtiter plates in final volume of 50 μl of nutritient medium (RPMI 1640 with l-glutamine, HEPES, antibiotics. After 20 hours, five different concentrations of 8-Cl-cAMP (in the range 2-74 μM) in nutritient medium, sometimes with fresh or heat (56°C, 30 min), inactivated fetal bovine serum, FBS, otherwise in the presence of fresh or heat inactivated human sera Hs, obtained from healthy volunteers were added to the group of wells, but not in corresponding control wells, where only nutrient medium was added. Final serum concentrations in samples were 10%. In samples used for study of combined action of IBMX and 8-Cl-cAMP, twenty hours after the cell seeding, IBMX was, 1 hour earlier, added to cells than 8-Cl-cAMP. Dipyridamole was added 2hrs before 8-Cl-cAMP. All analyses were done in triplicate. Nutritient medium with or without all cor-

responding addition(s), but without target cells, was used as blank, in triplicate too.

Treatment of K562 cells. K562 cells were seeded in triplicate into 96-well microtiter plates in final volume of 0.1 ml of nutritient medium (RPMI 1640 with L- glutamine, HEPES, antibiotics, sometimes with 10% fresh or heat (56°C, 30 min) inactivated fetal bovine serum, FBS, otherwise in the presence of 10% of fresh or heat inactivated human sera Hs, obtained from healthy volunteers IBMX, or dipyridamole, or 8-Cl-cAMP were added to the wells with the same schedule as to the HeLa cells.

Determination of HeLa, B16, and K562 cell survival. Cell survival was determined by MTT test according to the method of Mosmann (38) modified by Ohno and Abe (39), 72 hrs after the drug addition. Briefly, 20 µL of MTT solution (5 mg/ml PBS) were added to each well. Samples were incubated for further four hours at 37°C in 5% CO_2 and humidified air atmosphere. Then, 100 µL of 10% SDS in 0.01M HCl were added to the wells. Optical density (OD) at 570 nm was read the next day. To get cell survival (%), optical density at 570 nm of a sample with cells grown in the presence of various concentration of the agent (or agents) OD, was divided by control optical density ODc, (the OD of cells grown only in nutritient medium). (It was implied that OD of blank was always subtracted from OD of a corresponding sample with target cells). Concentration IC_{50} was defined as the concentration of a drug required to inhibit cell survival by 50%, compared with vehicle-treated control.

Results

The influence of different 8-Cl-cAMP concentrations on the survival of HeLa cells grown in nutritient medium with 10% fresh human sera (depending on the number of seeded cells per well) is shown in Figure 1. It could be seen that the extent of antiproliferative 8-Cl-cAMP action is boosted if a lower number of cells per well is seeded. So, the following experiments, where comparisons of different experimental conditions on 8-Cl-cAMP growth inhibitory action was studied, were always done with the same number of seeded cells.

IC_{50} concentrations of 8-Cl-cAMP for HeLa cells grown in the presence of 10% fresh or heat inactivated, different human sera, as well as their ratios are shown in Table I. Similar data obtained for K562 cells are presented in Table II. IC_{50} of 8-Cl-cAMP for K562 and B16 cells grown in the presence of 10% FBS are illustrated in Table III. Results obtained in this set of exper-

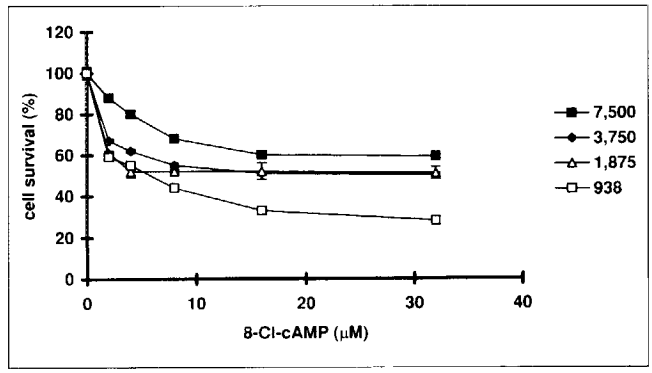

Fig. 1 - Survival of HeLa cells (%) 72 hrs after the agent action, given as the function of the various concentrations of 8-Cl-cAMP and number of seeded cells per well ($7.5x10^3$ cells per well; $3.75x10^3$ cells per well, $1.88x10^3$ cells per well and $0.94x10^3$ cells per well). Results are mean of triplicate.

Table I - The IC_{50} for the antiproliferative action of 8-Cl-cAMP towards HeLa cells in the presence of various, fresh or heat inactivated, human sera

N]	$IC_{50\ Hs}$ (µM)	$IC_{50\ Hs\ in}$ (µM)	$F(IC_{50\ Hs}/IC_{50\ Hs\ in})$
1	4.5	21	5.1
2	4.0	15	3.8
3	4.0	19	4.8
4	4.0	9	2.2
5	4.8	–	–

Table II - The IC_{50} for the action of 8-Cl-cAMP on K562 cells survival in the presence of various, fresh or heat inactivated, human sera

N]	$IC_{50\ Hs}$ (µM)	$IC_{50\ Hs\ in}$ (µM)	$F(IC_{50\ Hs}/IC_{50\ Hs\ in})$
1	7.5	44	5.9
2	16.5	52	3.15

Table III - The IC_{50} for the action of 8-Cl-cAMP towards K562 cells survival (N/N_c) in the presence of various, fresh or heat inactivated, fetal bovine sera

	$IC_{50\ FBS}$ (µM)	$IC_{50\ FBS\ in}$ (µM)	$F(IC_{50}/IC_{50\ FBS\ in})$
K562	16	65	4
B16	8	–	–

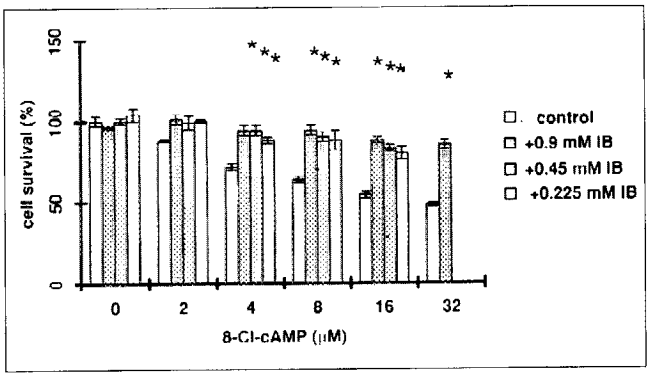

Fig. 2 - The effect of different concentrations of IBMX in the presence of 10% fresh human sera in nutritient medium, on HeLa cell survival (%), determined 72 hrs after the agent action, as the function of the various concentration of 8-Cl-cAMP. IBMX was applied to cell culture 1 hr before 8-Cl-cAMP. Results are mean of triplicate. * $p < 0.001$

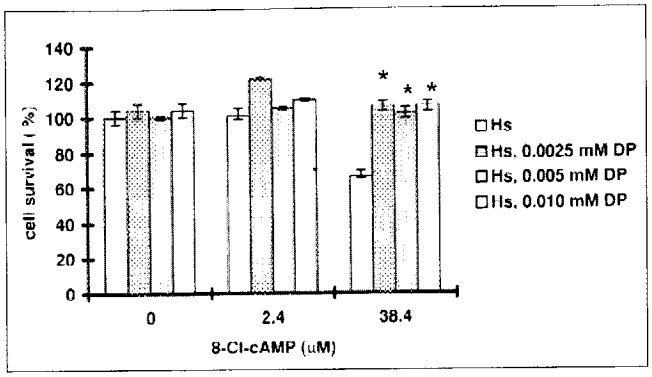

Fig. 4 - The effect of 2.5 mM; 5 mM and 10 mM dipyridamole, DP, on the survival (%) of HeLa cells grown in the presence of various concentration of 8-Cl-cAMP, with 10% fresh human sera (Hs) in nutritient medium determined 72 hrs after the agents action. DP was applied to cell culture 2 hrs before the 8-Cl-cAMP.

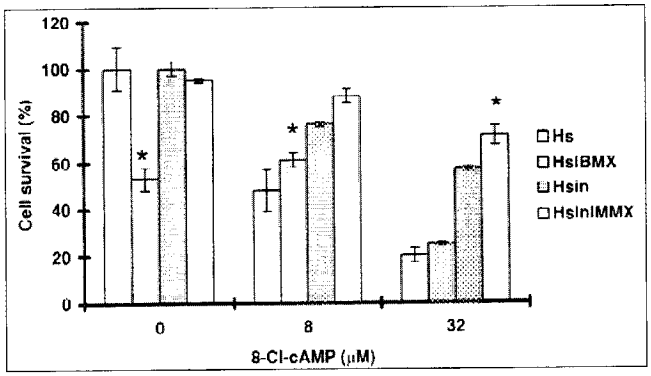

Fig. 3 - The effect of IBMX (0.36 mM) in the presence of 10% fresh Hs, or heat inactivated human sera (Hs in) in nutritient medium, on K562 cell survival (%), determined 72 hrs after the agents action, as the function of the various concentrations of 8-Cl-cAMP. IBMX was applied to cell culture 1 hr before 8-Cl-cAMP. Results are mean of triplicate. * $p < 0.001$

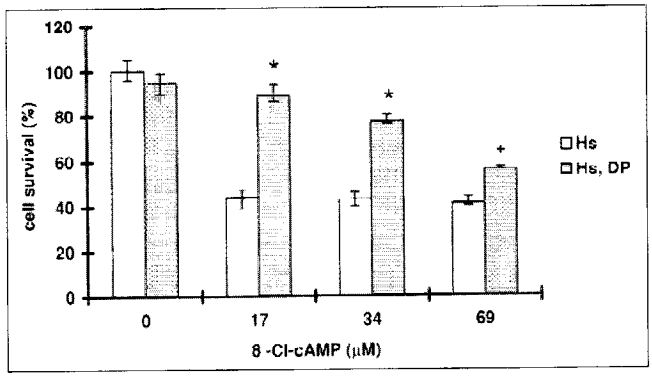

Fig. 5 - The effect of 10 mM dipyridamole (DP) on the survival (%) of K562 cells grown in the presence of various concentration of 8-Cl-cAMP, in the presence of 10 % fresh human sera (Hs) in nutritient medium, determined 72 hrs after the agents action, DP was applied to cell culture 2 hrs before the 8-Cl-cAMP.
* $p < 0.001$

iments showed that there was some termolabile factor(s) in Hs, as well as in FBS, that activated (or permitted) the antiproliferative 8-Cl-cAMP action to investigated cell lines. IC_{50} for various fresh human sera were not so reciprocally variable for HeLa cells, although it was shown that they could be different for leukaemia K562 cell line.

The effect of phosphodiesterase inhibitor IBMX in the presence of fresh human sera, on 8-Cl-cAMP action on HeLa and K562 cell survival is presented in Fig. 2 and Fig. 3, respectively. It could be seen that IBMX alone suppressed survival of cells, when fresh human sera were present in nutritient medium. It could also be seen, that survival of HeLa and K562 cells, pre-treated with IBMX and incubated with 8-Cl-cAMP, was higher than that of cells which were not pre-treated with IBMX.

The suppression of 8-Cl-cAMP antiproliferative action (in the presence of fresh human sera), induced by nucleoside transport inhibitor, dipyridamole, pre-treatment on HeLa and K562 cell survival is shown in Fig. 4 and 5. Results showed that there were no differences between the responses of HeLa or K562 cells to the combined action of these two drugs.

The survival of HeLa cells, pretreated with 8-Cl-cAMP and treated with tiazofurin, is presented on

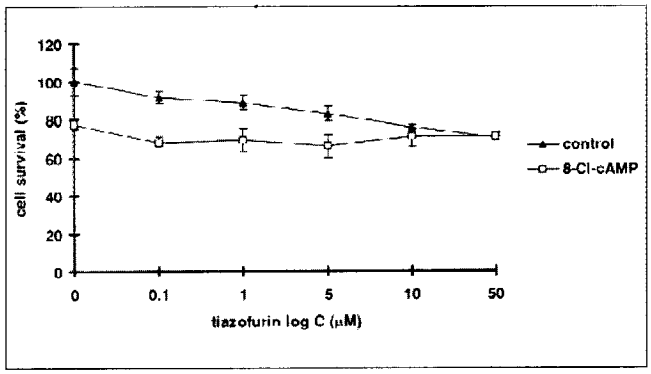

Fig. 6 - Survival (%) of HeLa non-treated cells, or pre-treated with 4mM 8-Cl-cAMP determined 72 hrs after the agents action as the function of the various concentration of tiazofurin. Results are mean of triplicate.

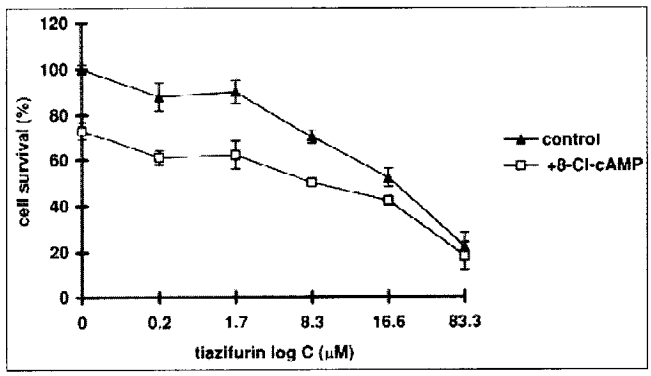

Fig. 7 - Survival (%) of K562 non-treated cells, or pre-treated with 4mM 8-Cl-cAMP determined 72 hrs after the agents action as the function of the various concentration of tiazofurin. Results are mean of triplicate.

Fig. 6 for HeLa cells and on Fig.7 for K562 cells. While an antagonistic action of 8-Cl-cAMP on antiproliferative tiazofurin action towards HeLa cells could be seen; such effect was not expressed on K562 cells.

Discussion

The antiproliferative 8-Cl-cAMP action was demonstrated on many malignant cell lines (11, 19, 21-23, 25), as well as on human xenografts (26, 27, 33). As soon as the phase I of clinical trial started, it was intriguing to see how the presence of different human sera (earlier experiments *in vitro* were done in the presence of fetal bovine sera) influences the cell growth inhibitory effect of the investigated drug. It was shown that IC_{50} of 8-ClcAMP was not so different when experiments were carried out in fresh FBS or Hs. Human serum heat inactivation led to the pronounced decrease in 8-Cl-cAMP activity to HeLa and K562 cells. A similar effect of FBS-heat inactivation on 8-Cl-cAMP action was observed on mouse melanoma and mouse friend leukemia cells, FLC (26) human promyelocytic cell line HL-60 (40), MCF-7 cells (41), as well as on normal and neoplastic mouse lung epithelial cells (35), indicating that some termolabile factor(s), that metabolically activates or permits 8-Cl-AMP action, exists in fresh human sera.

Our experiments clearly show that IBMX, in the presence of human serum, could partly suppress 8-Cl-cAMP action to HeLa and K562 cells, which is in accordance with the earlier reports showing that IBMX, in the presence of FBS, could suppress the action of 8-Cl-cAMP on normal and neoplastic mouse lung epithelial cells (35), on mouse melanoma and mouse friend leukemia cells, FLC (32), on MCF-7 cells (41), confirming that some cAMP-dependent PDE present in fresh HS could metabolically activate the prodrug 8-Cl-cAMP to drug 8-Cl-adenosine that exerted its antiproliferative action. IBMX could not only inhibit conversion of 8-Cl-cAMP to 8-Cl-adenosine, but can also rise the intracellular level of cAMP. CAMP complexed with unstable free RI (due to insufficient presence of catalytic C subunit after the 8-Cl-cAMP action) (35) could protect it from hydrolysis. So, the level of RI must not be downregulated and, conversely, cell growth must not be inhibited during the action of 8-Cl-cAMP in the presence of cAMP-dependent PDE in fresh human sera. It is worth noticing that a pretreatment of B16 cells with cAMP-dependent PDE inhibitor aminophylline also led to the decrease of 8-Cl-cAMP antiproliferative effect (results not shown).

Difference in 8-Cl-cAMP action depending on the number of cells seeded in well, highly pointed to the possibility that the investigated drug, or, in part, its non-cytotoxic metabolite could be constitutionally included in the control of cell growth. The data obtained on target cells, pretreated with nucleoside transport inhibitor dipyridamole, and then treated with 8-Cl-cAMP, supported the hypothesis according to which (29, 35) part of the antiproliferative 8-Cl-cAMP action could be pronounced through the constitutive intracellular drug (or its non-cytotoxic metabolite) action; it also pointed to the possibility that 8-Cl-cAMP could act to intracellularly located adenosine P site. This finding is supported by the data that 8-Cl-cAMP antagonized the antiproliferative tiazofurin action to HeLa cells, and possibly acts as an unsuitable precursor in the insufficient nucleoside pool obtained by the action of tiazofurin; however, it must be emphasized this strong antagonistic effect of 8-

Cl-cAMP was not observed for tiazofurin action on K562 cells.

In conclusion, results obtained in this work indicate that in the presence of human sera the antiproliferative 8-Cl-cAMP action towards human cervix carcinoma and myelogenous leukemia cells is completely pronounced. Therefore, this drug could be considered for the treatment of cervical carcinoma, malignant melanoma or myelogeneous leukemia, if the results of experiments on animal models would be in agreement with the data obtained.

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